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Title: AUTOMATED 2-DIMENSIONAL ANALYSIS OF BIOLOGICAL AND OTHER SAMPLES

FIELD OF THE INVENTION

The present invention is concerned with chromatographic materials and methods. More particularly, the present invention is concerned with high-resolution separation and high sensitivity detection of proteins and other biological samples.

BACKGROUND OF THE INVENTION

The word *proteome* was coined in 1995 to refer to the total protein complement of a genome. The human genome encodes roughly 100,000 genes, corresponding to a similar number of proteins. Not all genes are expressed in all tissues: roughly 10,000 proteins are found in any particular cell. The fraction of the proteome that is expressed by an organism varies between tissues and in response to the environment.

Single cell proteome analysis offers several important advantages. In particular, it is possible to monitor the distribution in the expression of protein markers correlated with cancer stage. Like ploidy measurements, the distribution of protein expression may have valuable prognostic value. Sub-populations of metastatic or therapy-resistant cells may be identified at an early stage to guide treatment.

Conventional proteome analysis is performed by two-dimensional gel electrophoresis and requires protein from roughly a million cells. Accordingly, an improved apparatus and method is required to improve resolution and sensitivity, as well as the number of samples which can be analyzed simultaneously.

Additionally, there is a new and developing field which here will be identified as "molecular cytometry". Cytometry is the study of individual cells and their contents, and is often used to characterize tumors. Molecular cytometry uses the chemical composition of the cell to affect that characterization. It has been suggested that protein markers could be found that "may allow us to identify not only the presence of cancer but perhaps

its malignant potential" (Peters, P., in *Prognostic cytometry and cytopathology of prostate cancer*, J.P. Karr, D.S. Coffey and W. Gardner, Eds. Elsevier; New York, 412-12 (1988)). Also, it has been suggested in 1995 "From a therapeutic standpoint, it is highly desirable to increase the diagnostic accuracy concerning grade of malignancy and treatment sensitivity of tumors. The analysis of total protein patterns of tumor cells from clinical material using 2-D electrophoresis provides new information, possibly leading to the development of new markers" (Franzen, B. et al. *Electrophoresis* 16, 1087-89 (1995)).

The present invention focuses on identification of these markers. Identifying the molecular alterations that distinguish any particular cancer cell from a normal cell will ultimately help to define the nature and predict the pathologic behaviour of that cancer cell, as well as the responsiveness to treatment of that particular tumor. By understanding the profile of molecular changes in any particular cancer it will become possible to correlate the resulting phenotype of that cancer with molecular events. Resulting knowledge will offer the potential for a better understanding of cancer biology; the discovery of new tools and biomarkers for detection, diagnosis, and prevention studies; and new targets for therapeutic development (Program Announcement Number PAR-98-067 of U.S. National Institute of Health).

The present inventors have realized that single-cell analysis provides several important advantages over analysis of cell extracts. First, single-cell proteome analysis provides information on both the mean and the distribution of protein expression among the cellular population. The distribution of DNA content within cancer cells is used as a prognostic indicator, where aneuploidy is taken as evidence of an aggressive cancer. The distribution of protein marker concentration may provide similar diagnostic and prognostic information.

Secondly, and similarly, the response to a therapeutic agent may be monitored across a cellular population. It may prove that chemotherapy targets a particular subset of the cell population. If the

disappearance of this subset were monitored, therapy can be guided to target the surviving cell population.

Thirdly, ploidy of a cell may be correlated with its protein electropherogram. The present invention incorporates an image cytometry microscope in our system to measure DNA content of individual cells. Cells with anomalous DNA content or abnormal cytopathology can then be selected for study.

Fourthly, single-cell protein electropherograms may be correlated with cell cycle in cultured cells. The understanding of protein expression as a function of cell cycle will provide information on gene expression and protein turnover.

Fifthly, single-cell analysis allows the assay of micro-dissected tissues without contamination from connective tissue, erythrocytes, fibroblasts, etc. These nonmalignant tissues contribute to the protein electropherogram of large biopsies, making identification of tumor-specific markers more difficult. Single-cell analysis, with microscopic selection of the cell, ensures that only tumor material is analyzed.

Lastly, the technology used to analyze single cells can also be used to study the small samples generated by fine needle aspirant biopsies. Surgical biopsies will not be needed in most cases to obtain enough material for 2-D proteome analysis.

Two-dimensional gel electrophoresis is conventionally used to study proteomes. O'Farrell developed this classic technique almost 25 years ago (O'Farrell, P.H., *J. Biol. Chem.* 250, 4007-21 (1975)). A sample is homogenized and proteins are extracted and separated by tube isoelectric focusing. The gel is removed from the tube and placed on an SDS-PAGE gel, where the proteins are separated at right angles to the isoelectric focusing gel. After separation, proteins are stained to create a 2-D display of spots corresponding to the pI and molecular weight of the proteins. This manual technology is tedious and somewhat of an art. Components separated by 2-D electrophoresis can be identified by Edman protein sequencing, by mass spectrometry, or by immunoassay. Often, a partial amino acid sequence is

sufficient to identify the protein by comparison with databases.

Column switching technology is well established in both gas and liquid chromatography for analysis of complex mixtures. In the simplest system, a fraction containing the component of interest is captured as it elutes from the first column; this fraction is subjected to additional chromatographic separations to achieve the desired level of purity.

These multidimensional separation techniques can be expanded so that the second column sequentially separates all fractions from the first column. These 2-D techniques are particularly useful when characterizing extremely complex samples. An early report used two successive chromatographic steps to purify peptides generated from the proteolytic digest of a human immunoglobulin (Yamamoto, H., Manabe, T., Okuyama, T., *J. Chromatogr.* 480, 277-83 (1989); Takahashi, N., Takahashi, Y., Putnam, F.W. *J. Chromatogr.* 266, 511-22 (1983)).

Jorgenson and others have developed elegant multicolumn separations for proteins and peptides (Bushey, M.M. and Jorgenson, J.W. *Anal. Chem.* 62, 161-67 (1990); Larmann, J.P., Lemmo, A.V., Moore, A.W., Jorgenson, J.W. *Electrophoresis* 14, 439-47 (1993); Holland, L.A. and Jorgenson, J.W. *Anal Chem.* 67, 3275-83 (1995); Moore, A.W. and Jorgenson, J.W. *Anal Chem.* 67, 3448-55 (1995); Moore, A.W. and Jorgenson, J.W. *Anal Chem.* 67, 3456-63 (1995); Rose, D.J. and Opiteck, G.J. *Anal Chem.* 66, 2529-36 (1994)). These systems rely on various combinations of size exclusion chromatography, reversed-phase chromatography, and zone electrophoresis to characterize amines, peptides, and proteins. In the most sophisticated version, a mass spectrometer is used to identify components separated by a coupled ion exchange/reversed phase chromatography or size- exclusion/reversed-phase chromatography system (Opiteck, G.J., Lewis, K.C., Jorgenson, J.W., Anderegg, R.J. *Anal. Chem.* 69, 1518-24 (1997); Opiteck, G.J., Jorgenson, J.W., Anderegg, R.J. *Anal. Chem.* 69, 2283-91 (1997); Liu, Y.M. and Sweedler, J.V. *Anal. Chem.* 68, 3928-33 (1996)).

In Jorgenson's experiments, fractions that elute from a microbore chromatography column are sequentially injected into an

electrophoresis capillary, where overlapping components are resolved. Since electrophoresis is faster than the elution time of a chromatographic peak, all components from the chromatography column are sampled by the electrophoresis capillary. A 2-D electropherogram is reconstructed by plotting the electrophoresis separations next to each other. The appearance of the electropherogram is quite similar to a classic 2-D electropherogram, albeit generated by combining liquid chromatography with capillary electrophoresis.

The sequential separation offers several advantages over conventional 2-D electrophoresis, where fragments are separated simultaneously. Firstly, because fractions are detected sequentially, a sensitivity detector can be incorporated into the instrument. Secondly, the separation is automated; once the sample is injected, there is no further operator intervention.

The interface between the two columns is key to the performance of the system. Jorgenson has demonstrated an elegant flow-gated interface to couple a liquid chromatography column with a capillary electrophoresis column (Hooker, T.F. and Jorgenson, J.W. *Anal Chem.* 69, 4134-42 (1997); Lemmo, A.V. and Jorgenson, J.W. *Anal. Chem.* 65, 1576-81 (1993)). This interface uses a cross-flow of buffer to control injections of the eluted chromatographic fraction into a capillary zone electrophoresis (CZE) column. The effluent continually migrates from the HPLC capillary and is swept to waste by the cross-flow in the interface. To inject a fraction into the CZE capillary, the cross-flow of buffer is halted and a slug of HPLC effluent forms in the interface. Potential is applied from the buffer vial across the CZE capillary to the detection end of the capillary, which injects the HPLC effluent into the electrophoresis column. Electric field is applied across the electrophoresis capillary to separate the injected components. To avoid continuous injection of the HPLC components during the electrophoresis step, buffer flows through the interface, sweeping HPLC effluent to waste. Note that the flow-gated detector throws away most of the HPLC effluent, which is washed downstream to waste, and this leads to wastage of a large

part of the sample.

SUMMARY OF THE INVENTION

The present invention provides an apparatus and method to obtain high resolution separation and high sensitivity detection of proteins and other components contained in biological samples.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

In accordance with a first aspect of the present invention, there is provided an apparatus to provide separation and detection of components within a sample, the apparatus comprising a first separation means, an interface means, and a second separation means wherein the interface means links the first separation means and second separate means and a detector for detecting components subjected to the apparatus.

The apparatus can include a first high voltage power source connected across the first separation means and a second high voltage power source connected across the second separation means. Further, the first separation means can be a capillary electrophoresis system and the second separation means can be a sieving electrophoresis system.

Another aspect of the present invention provides an apparatus for providing high sensitivity detection of components of biological samples, the apparatus comprising: a first and second separation means, each selected from the group consisting of: isoelectric focusing electrophoresis systems, SDS polyacrylamide gel electrophoresis system, a free solution electrophoresis system, a micellar electrokinetic chromatography system, a reversed phase liquid chromatography system, a normal phase chromatography system. an ion exchange chromatography

system, and a size exclusion chromatography system; an interface chamber in which components separated according to said first means are mixed with a derivatizing agent prior to subjection to said second separation means; one of a first power supply and a first pump to perform the first separation means, and one of a second pump and a second power supply to perform the second separation means; and a detector.

Advantageously, the apparatus includes a plurality of first separation means, a plurality of second separation means, and a manifold providing a plurality of interface regions, each interface region providing an interface between a respective one of the first separation means and a respective one of the second separation means.

The manifold can comprise an inlet, for connection to buffer reservoirs and valve means permitting selective connection to a desired buffer reservoir; a channel network connecting the inlet to the plurality of interface regions, wherein each interface region comprises a port for connection to a respective one of the first separation means, a port for connection to a respective one of the second separation means and a third, waste port.

A further aspect of the present invention provides a method of separating and detecting components in a sample, the method comprising subjecting said sample to an apparatus which consists of high resolution separation and high sensitivity detection of components within a sample, the apparatus comprising a first separation means, an interface means, a second separation means and a detector for detecting components subjected to the apparatus, wherein the interface means links the first separation means and the second separation means, the method comprising passing the biological sample through the first separation means to achieve a first separation, passing the sample out of the first separation means into the interface means, separating the sample into fractions with the interface means, and separately passing each fraction through the second separation means.

The method can include providing an electric field across

each of the first separation means and the second separation means with a high voltage power source.

Yet another method provided by the present invention comprises providing high solution separation and high sensitivity detection of components in biological samples, the method comprising:

(a) passing a biological sample through a first separation means selected from the group consisting of: isoelectric focusing electrophoresis systems, SDS polyacrylamide gel electrophoresis system, a free solution electrophoresis system, a micellar electrokinetic chromatography system, a reversed phase liquid chromatography system, a normal phase chromatography system, an ion exchange chromatography system, and a size exclusion chromatography system;

(b) passing the sample out of the first separation means and separating the sample into fractions;

(c) passing each fraction separately through a second separation means selected from the group consisting of: isoelectric focusing electrophoresis systems, SDS polyacrylamide gel electrophoresis system, a free solution electrophoresis system, a micellar electrokinetic chromatography system, a reversed phase liquid chromatography system, a normal phase chromatography system, an ion exchange chromatography system, and a size exclusion chromatography system;

(d) detecting components of the sample leaving the second separation means with a detector, wherein the method includes applying voltages across said first separation means and said second separation means.

The method can further comprise providing a plurality of first separation means and a plurality of second separation means and a plurality of interface means, with each interface means providing a link between a respective one of the first separation means and respective one of the second separation means, and wherein the method further comprises, for each of the first and second separation means linked by a respective interface means, passing a biological sample through the first separation

means to achieve a first separation, passing the sample out of the first separation means into the respective interface means, separating the sample into fractions with the interface means and separately passing each fraction through a second separation means.

Then the method can include providing all the interface means in a common manifold, providing the manifold with an inlet, for connection to buffer reservoirs, a valve means permitting selective connection to a desired buffer reservoir, a channel network connecting the inlet to the plurality of interface regions, providing each interface region with a port connected to a respective first separation means, a port connected to a respective second separation means and a third, waste port, wherein the method comprises providing a plurality of buffer reservoirs connected to the inlet of a manifold and operating the valve means to connect a selected buffer reservoir to the manifold, whereby the same buffer reservoir is connected to all the interface regions and similar processing steps occur simultaneously in the interface regions.

The method can also include providing a planar surface with a plurality of immobilization sites for capturing cells, providing the first separation means with capillary tubes having inlet ends and mounting the inlet ends in an array corresponding to the location of immobilization agents on the planar surface, and wherein the method further comprises providing a biological sample on the planar surface, whereby at least one cell is captured by each immobilization agent site, aligning the inlet ends of the capillary tubes of the first separation means with the immobilization agent sites, and drawing the cells into the capillary tubes of the first separation means, for effecting a first separation in each capillary tube.

Finally, a further aspect of the present invention provides a manifold for use in separation and detection of components for the sample, the manifold comprising an inlet, for connection to buffer reservoirs and valve means permitting selective connection to a desired buffer reservoir; a channel network connecting the inlet to a plurality of interface regions; wherein each interface region comprises a port for

connection to a first separation means, a port for connection to a second separation means and a third, waste port.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings which show preferred embodiments of the present invention and in which:

Figure 1 shows a schematic representation of an instrument of the present invention;

Figure 2 shows a schematic representation of a flow interface of fluids in an instrument of the present invention;

Figure 3 shows a raster image to generate a two dimensional image of data from an instrument of the present invention;

Figure 4 illustrates a schematic representation of a multi-capillary version of an instrument of the present invention; and

Figure 5 shows a perspective view of a 2-D sheath flow cuvette detector.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides an apparatus and method to obtain high solution separation and high sensitivity detection of proteins or other components contained in biological samples. According to this embodiment of the present invention, there is provided a first dimension separation, preferably performed by capillary electrophoresis or by chromatographic means. According to the invention, this first separation technique divides the sample into a number of fractions where each fraction may contain one or more components.

The invention further provides that each fraction is directed to an interface chamber. Once the fraction is introduced into the interface, flow is interrupted from the first-dimension separation means and the component(s) within each fraction are mixed with a derivatizing reagent, preferably a fluorescent-labelling reagent. Once labelled, the

component(s) are then subjected to a second-dimension separation. This second-dimension separation means, further separates the components within the fraction from the first-dimension separation means. According to a preferred embodiment, the two separation means should separate components based on different properties of the components. Classically, isoelectric focusing and SDS-polyacrylamide gel electrophoresis are used to separate proteins; the isoelectric focusing technology separates proteins based on differences in their isoelectric point while gel electrophoresis separates components based on size. Alternatively, an electrophoresis technique such as SDS-polyacrylamide gel electrophoresis, isoelectric focusing, isotachophoresis, or free solution electrophoresis could be combined with a chromatographic separation technique such as reversed phase chromatography, normal phase chromatography, or ion exchange chromatography. In general, an electrophoresis technique is preferred for the first- dimension separation because its flow can be stopped quickly simply by removing the electric field across the capillary. In contrast, chromatographic systems require a long time to depressurize, and their flow is much more difficult to stop.

Components are detected as they migrate or elute from the end of the second-dimension separation means, which is preferably a column. Laser-induced fluorescence is preferred, but other techniques such as absorbance, chemiluminescence, electrochemical, or mass spectrometric may be used. Fluorescence and mass spectrometry are believed to be an advantageous combination; fluorescence provides high sensitivity and mass spectrometry provides additional information to assist in the identification of unknown components. Component(s) that migrate or elute from the first-dimension separation means are derivatized for detection (preferably fluorescently labelled) before analysis by the second separation means. That approach is most useful when the second dimension separation means does not discriminate significantly between components with one or more label attached; it is difficult to control the extent of labelling with high precision. As an alternative, a labelling reagent

can be added after the second dimension separation column but before detection. Post-column reactions are less desirable because more reagent is required, as components are separated by the second-dimension separation means can remix within the post-column reaction chamber. Also, the reaction time is often limited in post column reactors because the flow is not interrupted; as a result, the reaction is not as efficient, less fluorescent product is generated, and sensitivity is lower. Finally, the reaction reagents are present in the detector with the sample, leading to higher background signal; the second-dimension separation column separates reagent and products with the method described above.

This preferred embodiment of the invention uses isoelectric focusing electrophoresis and sieving electrophoresis as the two separation means. While others can be used, this combination is most familiar to the biochemical community in the classic slab-gel format for analysis of protein mixtures. Those skilled in the art will readily appreciate that other separation techniques can also be used. For example, the techniques of SDS-gel, free zone electrophoresis, isoelectric focusing, normal phase chromatography, reversed phase chromatography, ion exchange chromatography, or size exclusion chromatography can be combined in any combination, preferably with an electrophoretic first dimension.

Single Cell Analysis

The inventors have been involved in single-cell analysis for the past three years. Most of their work has focused on the analysis of oligosaccharide metabolizing enzymes (J.Y. Zhao, et al. *Glycobiology* 4, 239-242 (1994); Zhang, Y. et al. *Anal. Biochem.* 227, 368-76 (1995); Le, X.C. et al. *J. Chromatogr.* 716, 215-20 (1995); Chan, N.W.C. et al. *Glycobiology* 5, 683-88 (1995); Le, X.C. et al. *J. Chromatogr.* 781, 515-22 (1997); Le, X.C. et al. *Glycobiology*, in press). In these experiments, the inventors have developed efficient technology to inject a single eucaryotic cell into a fused silica capillary, followed by lysis and electrophoretic analysis of the cell's content with high sensitivity laser-induced fluorescence detection.

The inventors have used an Olympus inverted microscope in their experiments, equipped with a set of x-y-z hydraulic micromanipulators. A transparent capillary holder has been constructed and interfaced with the manipulators. This holder allows the observation of the cell by transmission microscopy while placing the capillary over the cell. This capillary holder has features that allow the simple injection of a cell onto the capillary, a buffer reservoir for electrophoresis, and a pressurized chamber to flush the capillary with a sodium hydroxide solution between runs.

A drop of cell suspension in phosphate-buffered saline solution is placed on a microscope slide. Cells are observed with an inverted microscope in phase-contrast mode. Once a cell is selected for analysis, micromanipulators are used to lower a capillary (20 mm diameter) with a flat tip over the cell. A computer-controlled pulse of vacuum is applied to the distal end of the capillary, which reproducibly draws the cell and a small slug of supernatant solution into the capillary. Based on the migration time of fluorescent components of the cell, it is estimated that injection draws the cell about 0.5 mm into the capillary with a relative precision of 0.05 mm.

Once the cell is drawn into the capillary, the cell must be lysed to release its contents for further analysis. The capillary tip is placed in a buffer-filled vial, which was then placed in an ultrasonic bath. This method is quite efficient in lysing the cell. However, the transfer of the capillary and vial from the microscope to the ultrasonic bath is slow and not particularly reproducible. It is difficult to avoid the formation of a siphon during this transfer, which can displace the cell and its contents within the capillary.

Human cancer cells are lysed within 30 seconds of contact with an SDS buffer. The action of the surfactant is rapid and reproducible. A 10 mM SDS, 50 mM phosphate buffer was used for the separation. The capillary is filled with this buffer before the injection of a single cell. After injection of a cell, the surfactant quickly diffuses into contact with the cell during the incubation, leading to efficient lysis.

Single-cell proteome analysis has one disadvantage compared with flow cytometry: analysis of thousands of cells will be difficult. On the other hand, the huge amount of data generated from each cell will more than make up for the modest data acquisition rate. To optimize the value of information that is generated, a microscopic inspection of a biopsy will be performed and selected for subsequent proteome analysis in the first generation instrument.

A fine-needle aspirant biopsy is spread on a microscope slide and viewed with an inverted microscope. The cells are often clumped when observed under the microscope. The cells are dispersed as a dilute suspension. It is important that the cells are not in close proximity, so that the position of adjacent cells is not disturbed by the selection process. Also, cellulose will be added to the suspension buffer to ensure that the cells do not move before injection.

The microscope stage is provided with stepper-motor driven in micrometers. These micrometers are controlled with a joystick, so that a user can scan the image field in precise increments. A foot-button will be depressed to indicate the location of a cell that is to be analyzed. A computer will record the position of that cell. Once a set of cells has been chosen for analysis, an injection/electrophoresis program will be activated. This program will drive the micrometers so that each cell is moved, in succession, to the center of the field-of-view of the microscope. The cells are photographed with a CCD camera mounted on the microscope; the image is digitized and stored so that it can be used later for comparison with the proteome data.

The cells are then treated with a DNA stain before inspection and the DNA content of the cell will be estimated based on fluorescence from the dye. The use of Hoesch 33342 fluorescent dye (Molecular Probes, Eugene, Oregon, USA) is preferred, which is permeant to living cells and which does not require that cells be fixed before staining. Also, the dye is only excited in the UV, so that no bleaching will occur during preliminary inspection with phase-contrast illumination. The

microscope will be equipped with a photomultiplier tube to measure fluorescence in a 10-μm diameter region in the center of the microscope's field-of-view.

The micrometer position will be automatically adjusted to maximize fluorescence from the cell, ensuring that the cell is precisely centered in the field-of-view. In our microscope, the DNA stain is photobleached over a 1-minute illumination period. We anticipate that it will take 10 seconds to adjust the cell's position to maximize the fluorescence signal and capture the peak intensity. The DNA fluorescence will be used to determine the cell's ploidy. If appropriate, only cells with specific ploidy will be taken for subsequent injection into the electrophoresis capillary.

Each cell that matches the specified criteria is injected into the separation capillary. Both flat-tipped and etched-tip capillaries have been investigated for injection. The capillary will be positioned precisely over the center of the field of view of the microscope, which will correspond to the location of the cell of interest. The capillary will be lowered to the microscope slide to surround the cell. A solenoid at the distal end of the capillary will be opened for 1 second, exposing that end of the capillary to the house-vacuum and drawing the cell into the capillary. Of course, for multiple capillaries (detailed below) all other capillaries and reservoirs will have their flow blocked at this point to ensure successful cell injection.

The individual capillary or group of capillaries will then be moved to a set of buffer reservoirs. If the cell was pretreated with FQ to label cytoplasmic proteins for 1-D electrophoresis of if the cell is to be subjected to 2-D electrophoresis, the first reservoir will contain only a TWEEN-20 solution to lyse the cell. Unlike SDS, the non-ionic detergent does not interfere in subsequent isoelectric focusing electrophoresis. If all the cell's proteins are to be labelled on-column before a 1-D separation, the first reservoir will contain both the lysing reagent and the derivatizing reagents. A nanoliter of this solution will be injected into the capillary, and

cell lysis is expected to be complete within 30 seconds. The capillary tip will then be placed in an appropriate running buffer. If 1-D free-solution electrophoresis is performed, that buffer will be a basic SDS-phosphate buffer. If capillary isoelectric focusing is the first dimension of a 2-D analysis, then an acidic anolyte will be used as the buffer.

Once the separation is complete, the next cell will be moved to the microscope's field-of-view, a photograph will be taken, fluorescence will be measured, and the cell will be analyzed by electrophoresis. The procedure will be repeated for all of the cells that were specified by the pathologist. To prevent evaporation of the cell suspension buffer, the cell chamber will be kept in a constant humidity chamber.

Single Capillary Embodiment

Referring first to Figure 1, there is shown an apparatus 10, including first dimension separation column 12 and a second dimension separation column 14. In known manner, the two separation columns 12, 14 are capillaries, with an internal diameter of 10 to 75 μm.

The first dimension separation 12, as noted above, uses isoelectric focusing. For this purpose, the column 12 is filled with a mixture of ampholites. A first high voltage source 16 is connected, as indicated at 18 to one end of the column 12 and as indicated at 20 to an interface 22. The interface 22 is detailed further below, and is connected to both the columns 12, 14 as shown.

A second high voltage source 24 is connected to the interface 22, as indicated at 26, and as shown at 28, to the other end of the second dimension column 14.

The interface 22 is shown in detail in Figure 2 and is constructed from an inert, non-conducting material, such as a high quality plastic. Four holes 30 are machined in the plastic and are connected by bores 32 to form a cross configuration. As indicated schematically, the holes are preferably equipped with a ferrule system or some other means of sealing the capillary tubing in place. The dimensions of the holes 30 and bores 32

should be small, with holes 30 being preferably less than 1 mm in diameter and the bores 32, 0.2 mm in diameter. The capillary columns 12, 14 are shown schematically in Figure 2, connected to the high voltage sources 16, 24. The connections 20, 26 of the high voltage sources are shown here connected to a drain or waste line 34 for a buffer solution, that is also being shown in Figure 1.

A buffer inlet 36 is connected to pneumatically controlled switching valve 38, which can be switched between four positions. Three of these positions provide a connection to one of three buffer reservoirs 40, 42 and 44. A fourth position connects the valve 38 to a blocked flow port 46.

The buffer reservoirs 40, 42 and 44 are placed above the pneumatic valve 38, so that flow from the buffer reservoirs can pass through the interface 22 by gravity. The valve 38 is pneumatically switched to avoid electric shock hazard from the potential applied during the electrophoresis steps. Similarly, the buffer reservoirs 40, 42 and 44 should be enclosed safely in an interlocked equipped chamber to avoid accidental contact during the electrophoresis steps.

In the apparatus 10, a sample is injected by placing it in solution in contact with one end of the capillary 12 and applying a brief electric field pulse. Alternatively, pressure may be used to fill a portion of the capillary 12 with sample. Once the sample has been applied, the sample is replaced with a running buffer (not shown) and electric field is applied by the source 16 across the first-dimension separation column 12; separation is thereby performed. According to the technique of isoelectric focusing which is known by those skilled in the art, the capillary 12 is filled with a mixture of ampholites which are complex mixtures of compounds with both weak acid and weak base functional groups. Ampholites can be mixed with the sample and the mixture used to fill the capillary 12. Alternatively, they can be used to fill the majority of the capillary and the sample may be injected later.

Once the capillary 12 is filled with sample and ampholites, the sample-end of the capillary is replaced with a sodium hydroxide (or

other appropriate base) solution, as the running buffer, and the interface 22 is filled with phosphoric acid (or other appropriate acid) solution from an appropriate one of the reservoirs 40, 42 and 44. The anode of the high voltage power supply 16 is connected to the acid end and the cathode is connected to the base end. A sufficient amount of voltage is applied to cause a pH gradient to form in the capillary 12. The proteins contained in the sample(s) move to a location on the capillary where the pH equals the protein's isoelectric point. The achievement of focusing is correlated with a drop of current across the capillary; when the current reaches about 10% or less of its initial value, the voltage supply 1 is turned off.

During the isoelectric focusing step, the interface 22 is filled with acid solution from reservoir 40 (Figure 2). To minimize the build-up of electrolysis products, this acid solution can flow continuously at a low rate (less than 10 mL/hour) during the isoelectric focusing step. Electric field is applied across the isoelectric capillary 12.

Mobilization of fraction to interface

The separated fractions are successively mobilized from the first dimension separation capillary into the interface. While several methods to mobilize the focused proteins will now be described, it will be readily apparent to those skilled in the art that these methods are in no way limited to those described. Other effective methods of mobilization are also with the scope of the present invention.

First, in according to a preferred embodiment, the interior of the first dimension separation capillary 12 is coated with a neutral material to reduce electro-osmosis and to reduce adsorption of proteins to the capillary interior. In this case, the proteins can be mobilized from the first dimension separation capillary by several means. For example, low pressure can be applied to the sample end of the first dimension separation capillary. In a basic approach, the sample end of the first dimension separation capillary may be raised by several centimetres above the level of the interface 22 and gravity will create a siphon to cause the sample to flow from the first dimension separation capillary into the interface 22. The

sample end of the first dimension separation capillary 12 can be lowered to cause the flow to cease after the appropriate fraction of the first dimension separation capillary's contents have flown into the interface 22. It is desirable to apply electric field during this step to keep the proteins focused in sharp bands. The electric field can be removed once the aliquot has been delivered to the interface 22.

According to an alternative embodiment, a chemical means can be used to urge a fraction of the first dimension separation capillary's contents into the interface. According to this embodiment, the acid-containing solution at the interface end of the first dimension separation capillary 12 is replaced with an appropriate basic buffer made from for example, a dilute sodium hydroxide solution. This change in pH of the solution causes the contents of the capillary to migrate toward the interface when an electric field is applied across the first dimension separation capillary.

According to yet another embodiment, if the interior of the first dimension separation capillary 12 is not treated with a neutral coating, then electro-osmosis will drive analyte from the capillary during the focusing step. According to the embodiment, the polarities would be reversed in figure 1 to ensure that the interface is at basic pH.

Once isoelectric focusing is completed, it is necessary to mobilize and label fractions from the isoelectric focusing capillary. A basic buffer is mixed with fluorescent derivatizing reagent (most derivatizing reagents require basic buffers) and is placed in the buffer reservoir 42. The interface should be flushed with the basic buffer/labelling reagent and then the flow from the reservoirs 40, 42, 44 should be blocked; no flow is desired during the step where fractions are mobilized from the isoelectric focusing capillary. Electric field is applied across the isoelectric focusing capillary for a short period (500-5000 V for 1-10 seconds), driving a fraction from the isoelectric focusing capillary into the interface to mix with the derivatizing reagent. Once the fraction has been introduced to the interface, the electric field is turned off.

Regardless of the method used, once a predetermined portion of the first-dimension separation column has migrated to the interface 22, removal of the electric field from high voltage supply 16 will terminate flow. Each aliquot or fraction that is transferred to the interface 22 would have a volume that is between about 0. 1% and about 5% of the volume of the capillary. Larger volumes result in mixing of components that migrate from the first dimension separation column while smaller volumes result in the analysis of many samples with a corresponding increase in total analysis time.

Labelling of fraction

Once the fraction of the first dimension separation capillary has been introduced to the interface, a plug of an appropriate derivatizing reagent is introduced into the interface. By appropriate derivatizing agent it is meant that the reagent reacts with the material separated by the first dimension separation capillary. For the analysis of proteins, this reagent could be 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), fluorescein sulfonyl chloride, ortho-phthaldialdehyde, or the like. Alternatively, if the compound is fluorescent, derivatization will not be necessary. It usually will be convenient to control the temperature of the interface; often, the interface should be heated to speed the reaction. Other derivatizing agents will be known to those skilled in the art.

The temperature of the interface can be increased to 50 °C to increase the speed of the fluorescence derivatizing reaction. Either the entire interface can be heated or just the portion corresponding to the intersection of the two holes.

Once the fluorescent derivatizing reaction is complete, the labelled sample must be injected into the SDS-gel capillary 14. Electric field is applied to the detection end of the SDS-gel capillary by the electrode or connection 28. The second electrode or connection 26 is in contact with the waste outlet 34 of the interface 22. Stacking occurs during injection. Most, if not all, of the labelled fraction from the isoelectric focusing capillary 12 is injected into the SDS-gel capillary 14 as a narrow plug.

Once the derivatizing reaction is complete, the interface's contents should be applied by injection or other appropriate means to the second dimension separation capillary.

Second Dimension Separation

Once the sample has been applied to the second dimension separation capillary 14, the interface 22 is preferably flushed with an appropriate separation buffer which is used in the second dimension separation. According to a preferred embodiment, this buffer is 5 mM HEPES containing 5 mM SDS (pH 8), again supplied from an appropriate one of the buffer reservoirs 40, 42 and 44.

The second dimension separation is performed by sieving electrophoresis. According to this embodiment, the capillary 14 is filled with an inert polymer. Those skilled in the art will appreciate what are appropriate polymers and that they include polymers such as polyacrylamide, dextran, or the like. Dextran is preferred because it has relatively low viscosity and can easily be pumped into and out of the second dimension separation capillary to rejuvenate that column as needed. Sieving electrophoresis is performed in the presence of a charged surfactant such as sodium dodecyl sulfate (SDS) or the like. This surfactant binds to the protein at the rate of roughly 1.4 g SDS to 1 g protein. This surfactant swamps the charge of the protein, creating a uniform charge along the protein length. When the protein passes through the sieving polymer-filled capillary, the migration time of the protein is proportional to the logarithm of molecular weight, with small proteins migrating first and larger proteins migrating later.

Once the sample is injected into the SDS-gel capillary, the SDS-gel buffer is passed through the interface from buffer reservoir 44. This buffer should flow at a low rate (less than 10 mL/hour) during the SDS-gel electrophoresis separation to minimize build-up of electrolysis products. High voltage is applied across the SDS-gel capillary to separate the fluorescently labelled proteins.

Separation is achieved by applying a potential across the

second dimension separation capillary 14 from the high voltage source 24. Preferably, at this time there is no potential across the first capillary 12. Proteins, or other components, that migrate from the second dimension separation capillary 14 are according to a preferred embodiment detected with a high sensitivity fluorescence detector (not shown). Other detection methods include absorbance, electrochemical, radiochemical, or mass spectroscopic means.

In this preferred embodiment detection will be achieved by a high sensitivity laser-induced fluorescence detector. This detector would be built from a sheath flow cuvette (Wu, S., Dovichi, N.J., *Journal of Chromatography* 480, 141-155 (1989)). Preferably, the laser wavelength should match the excitation wavelength of the labelled analyte and the emission wavelength should match the emission characteristics of the labelled analyte.

Upon completion of the SDS-gel separation, the capillary 14 should be flushed with fresh sieving media. The pneumatic valve 38 is turned to the blocked position 46, and fresh media should be pushed through the capillary from the detector.

A new fraction from the isoelectric focusing capillary 12 is mobilized into the interface, labelled, and separated by SDS-gel electrophoresis. This process is repeated until the entire contents of the isoelectric focusing capillary 12 have been analyzed.

Computer Reconstruction

The data resulting from the invention consist of sets of SDS-gel electropherograms: each electropherogram from successive fractions of the isoelectric focusing capillary. According to a preferred embodiment, these data can be presented as a raster image to generate a 2-dimensional map of proteins from the sample.

This is shown in Figure 3, where sample injection is shown at 50 and a detector at 52. The columns 12, 14 and interface 22 bear the same reference numerals. At 54, there is shown a schematic two-dimensional computerized reconstruction.

Laser-induced fluorescence detectors are used. These detectors, based on a sheath flow cuvette, provide good sensitivity. We use a 10-mW argon-ion laser beam at 488 nm for excitation; these lasers are reasonably rugged and provide good beam quality.

The instrument will be equipped with a two-colour fluorescence detector. This detector uses a dichroic filter to split fluorescence into two spectral bands. An interference filter is used to isolate fluorescence from FQ-labelled proteins in a 30-nm band centered at 630 nm. A second spectral channel will monitor fluorescence in a 25-nm band centered at 525 nm from fluorescein-labelled proteins. Fluorescence from the fluorescein-labelled internal standard proteins will be used as markers in the 2-D separation to correct for drift in migration times during and between assays.

Informatics will be a significant issue. Flicker, supplied by the National Institute of Health (USA), is public domain software that has been developed to visually compare 2-D electropherograms. For preliminary identification of proteins with significantly different expression levels in different samples, we will correct migration patterns based on the appearance of the fluorescein-labelled standard proteins. We will then convert the data to a GIF format and compare files using Flicker.

The QUEST (Monardo, P.J., Boutell, T., Garrels, Jl, Latter, Gl, *Computer Applications in the Biosciences*, 10, 137-143 (1994)) and Visage (distributed by MAYA Design Group, Pittsburgh, PA, USA) systems have also been developed for 2-D gel analysis. However, for quantitative identification of those components that differ between samples, Matlab, supplied by The Mathworks, Inc., Natick, Massachusetts, USA, is used to build our own image processing software. After migration patterns have been corrected based on the migration of standard proteins, a peak-finding algorithm will be used to locate and quantify each protein peak.

The concentration of each component will be measured and recorded from several samples, and the average and standard deviation of each protein's concentration will be determined. Last, a simple Student's

t-test or a Behrens-Fisher t-test will be used to identify those proteins whose concentration varies significantly between samples (Robinson *et al.* *Electrophoresis* 16, 1176-83 (1995)).

The 2-D capillary electrophoresis system will be used to analyze small amounts of proteins. Mass-spectrometric analysis of fractions will be difficult. To identify fractions, classic 2-D electrophoresis on a large cell extract is simultaneously performed. The same fluorescent protein markers used in the capillary system will be added to the sample. These fluorescent proteins will be used to normalize migration times so that the capillary 2-D and the slab 2-D electrophoresis data can be directly compared. Components of interest will be isolated from the slab 2-D gel, analyzed by mass spectrometry, and co-injected with the sample in the capillary system to confirm the identity of the component in the capillary data.

Multiple Capillary Embodiment

Referring to Figure 4, a second embodiment of the apparatus is indicated generally by the reference 60. This includes the reservoirs 40, 42 and 44, the pneumatic valve 38 and the blocked flow port 46 as for the first embodiment.

In the second embodiment 60, a manifold 62 is provided, replacing the interface 22 for the single capillary pair operation of Figures 1 and 2. As for the interface 22, the manifold 62 can be formed in plastic, glass, silicone or other suitable material. Conveniently, the manifold 62 is formed using photo lithographic techniques, to generate a set of channels, indicated at 64 to distribute reagents. The channel 64 can be from 10-200 micrometers in both width and depth.

The manifold 62 provides a plurality of interface regions 66, each having three connection ports, for connection to first and second columns and to a waste line.

A single interface region 66 is shown enlarged on the right hand side of Figure 4. At the top, this region 66 includes an inlet 68 for a buffer or reagent from one of the reservoirs 40, 42 and 44. A connection port 72 is provided for connection to a first capillary column 12, and a second

connection port 74 is provided for connection to a second column 14. A central port 76 is provided for connection to a waste outlet.

Thus, each interface region 66 can function as the interface 22 in the first embodiment. However, here, this multiple capillary detector 60 can be used to analyze signals from an array of capillaries. There may be as many as 96, or some multiple of 96, capillary-systems (that is pairs of capillaries 12, 14) operated in parallel.

Interface

Operation of a multiple capillary system, except for specific system dependent differences, is analogous to that of the single capillary version, described in the Detailed Description of Example 1. Briefly, analytes are introduced to an isoelectric focusing capillary, acidic buffer is introduced to the interface region, and an electric field is applied across the capillary. Once the components are focused to their isoelectric point, the electric field is turned off.

A fraction of the isoelectric focusing capillary's contents is mobilized by filling the interface region with basic buffer mixed with labelling reagent. An electric field is briefly applied across the isoelectric focusing capillary to mobilize a fraction to the interface.

The temperature of the interface may be raised to a level sufficient to increase the rate of the fluorescent labelling reaction. Once the reaction is complete, the labelled molecules are applied to the SDS-gel capillary by creating an electric field across the SDS- gel capillary.

Once the sample is applied to the SDS-gel capillary, the interface chamber is flushed with an appropriate buffer. Electric field is applied across the SDS-gel capillary to separate the components contained within the labelled fraction.

The second aspect of a preferred embodiment of a multiple capillary version of the present invention is a multiple capillary fluorescence detector. According to this embodiment of the invention, the detector may be similar to that described in US patents 5,439,578; 5,584,982; 5,741,412, or preferably 5,567,294, the contents of which are incorporated by

reference.

For multiple capillary operation, an array of antibodies is prepared on a microscope slide. The immobilization technology will be based on classic microphotolithography. A clean microscope slide is coated with a thin layer of photo-resist. The resist is illuminated with UV light through a mask that has 96 spots, 5-μm diameter, on 1-mm centers in an 8 x 12 array. The opaque spots will shadow the photo-resist, preventing photo-polymerization at those regions. The illuminated resist will polymerize and the unpolymerized resist will be washed from the 96 spots. The slide is then treated with an aminopropyl-silane reagent, which covalently binds to the glass surface. The photo-resist is removed with organic solvents, leaving an 8 x 12 array of 5-μm diameter pads of amino-propyl groups. Antibodies will be attached to the amino groups by use of classic diisothiocyanate chemistry. It is to be noted that other, and possibly better, choices of reagents could be made; for example, gold pads could be formed and antibodies immobilized by a thiol containing reagent.

A cell suspension is flushed across the treated slide to capture 96 cells. The 5-μm diameter pads are sufficiently small to ensure that a single eucaryotic cell will be captured at each pad. Once a cell has been captured, it will block adsorption of a second cell at that location.

Many different antibodies could be used to select a surface marker, isolating and concentrating specific cells. This technique can be used to immobilize an antibody to human blood-group A. This antibody will capture HT29 cells, which express the antigen on their cell-surface. Other capture reagents, such as lectins and aptamers could be used.

The microscope slide is held in a machined holder, which will locate the slide precisely on the microscope stage. Each pad will be sequentially centered in the field-of-view and a photograph will be taken using a CCD camera. The images will be stored for later inspection by a pathologist. Simultaneously, the UV fluorescence signal from each cell will be recorded to determine ploidy. We anticipate that 15 seconds will be required to image each cell, determine its ploidy, and move to the next cell;

less than 30 minutes will be required for the preliminary inspection of the cells.

A capillary array holder will be manufactured to hold a bundle of 96 capillaries in exact registration with the antibody pads that are formed on the microscope slide. The array of capillaries will be placed over the microscope slide and lowered to capture one cell within each capillary. A 3-second pulse of vacuum will be applied to the distal end of each capillary to draw the cell within the tube, and cell lysis will be performed as in the single capillary instrument.

The inventors have developed several high sensitivity multiple capillary fluorescence detectors for DNA sequencing, as disclosed in the U.S. patents identified above. The most recent form of detector is based on the 2-dimensional array of capillaries in a sheath flow cuvette.

Referring to Figure 5, there is shown a 2-dimensional sheath flow cuvette 70. A laser 72 provides a laser beam that is passed through cylindrical lenses 74 and 76, to generate an elliptically shaped beam. The laser is an argon-ion laser.

Radiation is collected end-on from the capillaries and passed through a camera lens 78, a bandpass filter 80, a prism 82 and further camera lens 84 to a CCD camera indicated at 86. This configuration shows that the fluorescence from each capillary is imaged onto a unique region of the camera chip. The prism 82 is included in the optical train to disperse fluorescence across the face of the camera, to enable recording of the complete spectrum from each capillary. The dispersion of the system is carefully tuned to ensure there is no spatial overlap of a fluorescence from each capillary.

Fluorescence is dispersed in the region between adjacent capillaries. A prism is used rather than a grating for two reasons. Firstly, the low dispersion of the prism simplifies the design of the optical system, ensuring there is no overlap. Secondly, the prism does not generate higher-order spectra that would interfere with the spectrum from adjacent capillaries.

The detector of Figure 5 continually monitors fluorescence from all capillaries and at 28 wavelengths across the fluorescence spectral band. The system offers three significant advantages compared to alternative designs. Firstly, the use of one-to-one image optics and eliminates optical losses associated with a demagnifying imaging system; the latter is found when a large linear capillary array is imaged onto a smaller photodiode array. Secondly, there is no loss in sensitivity due to a low duty cycle associated with a scanning detector across the array; a 96 capillary scanning instrument can observe fluorescence from any one capillary for no more than 1 per cent of the time. Thirdly, there is no loss in sensitivity due to sequential detection of fluorescence through a rotating filter wheel or prism array.

Rather than recording the fluorescence from 28 wavelengths simultaneously, fluorescence will be binned into two bands corresponding to fluorescein and FQ-labelled protein emission spectra. This binned data will occupy little computer memory.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.